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Instrumentation: Into the Black Box

I want first to clear up some confusion that results simply from words. A flow cytometer, despite its name, does not necessarily deal with cells; it deals with cells quite often, but it can also deal with chromosomes or molecules or latex beads or with many other particles that can be suspended in a fluid. Although early flow cytometers were developed with the purpose of sorting particles, many cytometers today are in fact not capable of sorting. To add to the semantic confusion, the acronym FACS is even applied to some of these non-sorting cytometers. Flow cytometry might be broadly defined as a system for measuring and then analyzing the signals that result as particles flow in a liquid stream through a beam of light. Flow cytometry is, however, a changing technology. Defining it is something like capturing a greased pig; the more tightly it is grasped, the more likely it is to wriggle free. In this chapter, I describe the components that make up a flow cytometric system in such a way that we may not need a definition, but will know one when we see it.

The common elements in all flow cytometers are

- A light source with a means to focus that light
- Fluid lines and controls to direct a liquid stream containing particles through the focused light beam
- An electronic network for detecting the light signals coming from the particles as they pass through the light beam and then converting the signals to numbers that are proportional to light intensity

- A computer for recording the numbers derived from the electronic detectors and then analyzing them

In this chapter I describe the way a flow cytometer shapes the light beam from a laser to illuminate cells; the fluid system that brings the cells into the light beam; and the electronic network for detecting and processing the signals coming from the cells. The next chapter will cover computing and analysis strategies for converting flow cytometric data into useful information.

ILLUMINATION OF THE STREAM

A laser beam has a circular, radially symmetrical cross-sectional profile, with a diameter of approximately 1–2 mm. Lenses in the cytometer itself are used to shape the laser beam and to focus it to a smaller diameter as it illuminates the cells. Simple spherical lenses can provide a round spot about 60 μm in diameter, with its most intense region in the center and its brightness decreasing out toward the edge of the spot. The decrease in intensity follows a Gaussian profile; cells passing through the middle of the beam are much more brightly illuminated than those passing near the periphery. By convention, the nominal diameter of a Gaussian beam of light refers to the distance across the center of the beam at which the intensity drops to 13.5% of its maximal, central value. Therefore, if a cell is 30 μm from the center of a nominal 60 μm beam, it will receive only 13.5% of the light it would receive at the center of that beam. At 10 μm from the center, the intensity is about 78% and, at 3 μm from the center, the intensity is 98% of the central intensity. Because intensity falls off rapidly at even small distances from the center of the beam, cells need to be confined to a well-defined path through the beam if they are going, one by one, to receive identical illumination as they flow through a round beam of light.

To alleviate this stringency, the beam-focusing lenses most frequently used in today's cytometers are a compromise; cylindrical lens combinations provide an elliptical spot, for example, 10–20 by 60 μm in size, with the short dimension parallel to the direction of cell flow and the longer dimension perpendicular to the flow (Fig. 3.1). By retaining a wide beam diameter across the direction of flow, an elliptical illumination spot can provide considerable side-to-side tolerance,

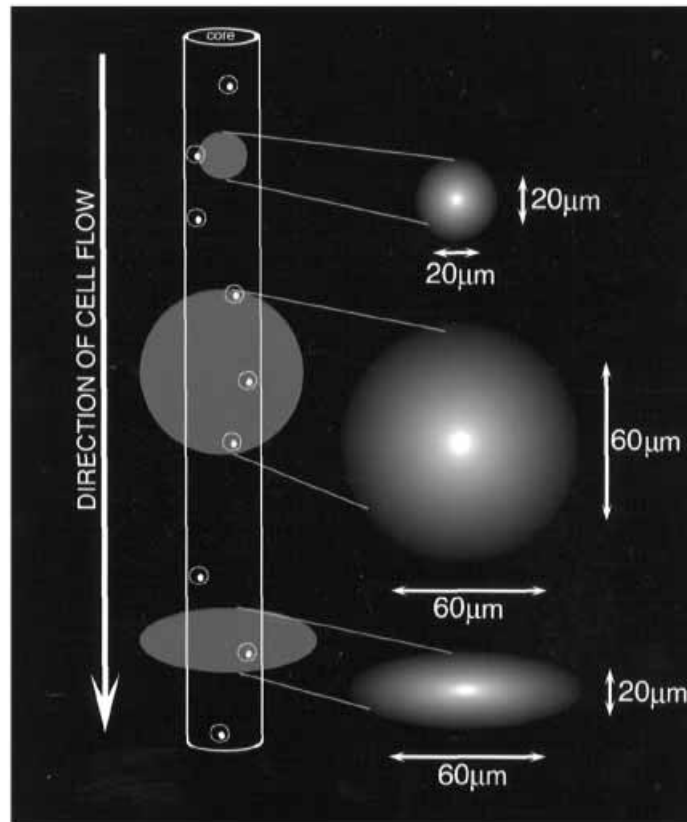


Fig. 3.1. Cells flowing past illuminating beams of different profiles. A beam with an elliptical profile (lower beam) allows cells to pass into and out of the beam quickly (avoiding the coincidence of two cells in the beam at the same time). In addition, it provides more equal illumination if cells stray from the center of the beam. The small circular beam at the top does not illuminate cells equally if they are at the edge of the stream core. The larger circular beam (in the middle) illuminates cells equally, but often includes multiple cells in the beam at the same time.

thus illuminating cells more-or-less identically even if they stray from the exact center of the beam; but at the same time this elliptical profile can provide temporal resolution between cells, illuminating only one at a time as they pass one by one into and out of the beam in its narrow dimension. The narrower the beam is, the more quickly will a cell pass through it—giving opportunity for the signal from that cell to drop off before the start of the signal from the next cell in line and avoiding the coincidence of two cells in the beam simultaneously. In

multilaser systems, each beam of light is focused in a similar way but at different points along the stream; a cell moves through each beam in sequence.

CENTERING CELLS IN THE ILLUMINATING BEAM

The fluidics in a flow cytometer are likely to be ignored until they go wrong. If they go wrong disastrously, they can make a terrible mess. If they go wrong with subtlety, they may turn a good experiment into artifactual nonsense without anyone ever noticing. On the assumption that the more disastrous problems can be solved by a combination of plumbing and mopping (both essential skills for flow cytometrists), I will concentrate on the more subtle aspects of fluid control. Nevertheless, the potential hazard of working at the same time with volumes of water and with a high-voltage source should never be far from the mind of anyone working with a water-cooled laser or with a sorting cytometer with high-voltage stream deflection plates.

The flow on a flow cytometer begins (Fig. 3.2) at a reservoir of liquid, called the *sheath fluid*. Sheath fluid provides the supporting vehicle for directing cells through the laser beam. The sheath fluid reservoir is pressurized, usually with pumped room air, to drive the sheath fluid through a filter to remove extraneous particles and then through plastic tubing to the illumination point. This sheath stream is usually buffer of a composition that is appropriate to the types of particles being analyzed. For leukocytes or other mammalian cells, this usually means some sort of phosphate-buffered saline solution. Other cells or other particles may have other preferences.

Different instruments employ different strategies for getting the sample with suspended cells into the sheath stream in the cytometer. Some instruments require cells to be in small test tubes that form a tight seal around an O-ring on a manifold. The manifold delivers air to the test tube, thus pushing the suspended cells up out of the test tube and through a plastic line to the sheath stream. Other instruments use a motor-driven syringe to remove a volume of sample and then inject it slowly into the cytometer. Depending on the instrument, there may be a greater or lesser degree of operator control over the rate of flow. The amount of pressure driving the sample through the system will affect the uniformity of alignment between the cells and

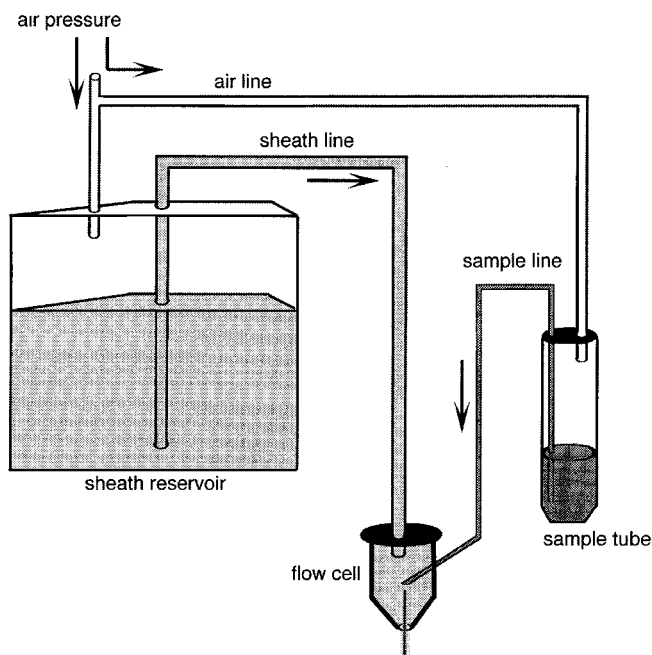


Fig. 3.2. The fluidics system, with air pressure pushing both the sample (with suspended cells) and the sheath fluid into the flow cell.

the illuminating laser beam as the cells move through the cytometer. Low pressure is less likely to cause perturbation of the stream profile and of the position of the cells within that stream. Empirically, if increasing the pressure on the sample causes undue broadening or wavering of signals, the pressure is probably excessive.

If cells flow too slowly through the cytometer, people start to make bad jokes about how microscopes cost less and are quicker. Because increasing the pressure may not be possible and, even if it is, is probably only a good idea within reasonable limits, the best way of getting cells to flow at reasonably fast rates is simply to make up the original sample with cells at a reasonably high concentration. A million cells per milliliter is often about right; 10^5 cells per milliliter is beginning to be low enough to test one's patience; 10^4 cells per milliliter is probably too low a concentration to be worth analyzing.

If you have few cells, make them up in a small volume (you will know how small a volume your system can handle). If the cells end

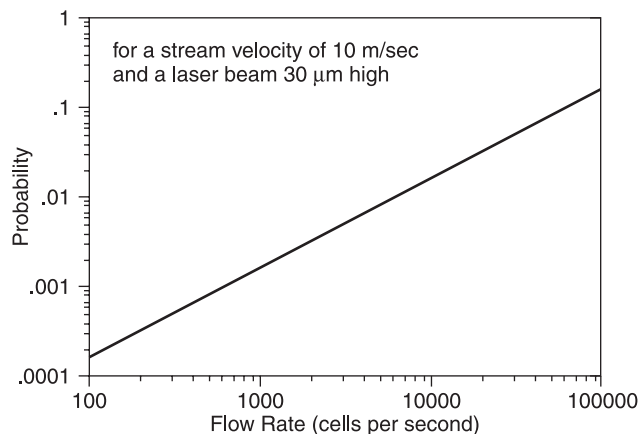


Fig. 3.3. The probability of an event recorded by the flow cytometer as a single “cell” actually resulting from more than one cell coinciding in the laser beam. For this model, the laser beam was considered to be 30 μm high and the stream flowing at 10 m per second.

up being too concentrated, they may flow too fast—but you can always dilute them on the spot and run the sample again. You may wonder why too rapid a flow is a source of problems. Faster seems as if it should always be better (especially around 5:00 PM). However, if cells are too close together as they flow through the laser beam, there may be difficulty separating their signals: A second cell may arrive in the illuminating beam before the preceding cell has emerged, and they will be measured together as if they were a single particle (with double the intensity). Figure 3.3 gives an indication of the probability of cells coinciding in the laser beam at different flow rates. Most cytometers seem to be quite happy looking at particles that are flowing at a rate of about 1000 particles per second.

Aside from concentration, another problem with samples is that the particles may be the wrong size. If they are too small, they may not be distinguishable from noise; nevertheless, bacteria and picoplankton and other bits and pieces of about 1 μm diameter or smaller are analyzable in at least some well-tuned cytometers. If the particles are too large, however, they will obstruct flow. If the fluid system is fully clogged, it may be difficult to get things flowing again; if it is only partially clogged, cells may flow but that critical alignment

between stream and light beam may be skewed, thus causing artifactual signals. Most experienced flow cytometrists recommend filtering any samples that are likely to contain large or clumped material before attempting to run them through the instrument. Nylon mesh of specified pore size works well (35 μm mesh is appropriate for most applications).

The exact size of particle that will be large enough to cause obstruction depends primarily on the diameter of the orifice of the nozzle or flow cell being used (usually between 50 and 250 μm). This brings us to the next stop downstream in our following of the flow in this flow cytometer. The terms *nozzle*, *flow cell*, and *flow chamber* derive from different engineering designs for the best way of delivering cells into the sheath fluid and thence to the analysis point where they are illuminated by the light. What we require is a method for keeping the cells in the center of the fluid stream so that they will pass through the center of the focused light beam and be uniformly illuminated. The flow chamber is the place in the cytometer where the cells from the sample join the fluid from the sheath reservoir. Within the flow chamber, the sample is injected into the center of the sheath stream; the combined sample and sheath streams are then accelerated as they move through a narrowing channel. This acceleration is critical to the precise alignment of cells, one at a time, in the laser beam. References at the end of this chapter will direct interested readers to mathematical discussion of the fluid mechanics related to this subject. For our purposes here, it will be enough to note that, as a result of considerations pertaining to laminar flow through a narrowing path, the sample with its suspended cells, after injection into the center of the sheath stream, will remain in a central core as it flows within the sheath stream out through the flow chamber.

The technical term for this is *hydrodynamic focusing*; flow of a sample stream within the center core of a sheath stream is called *coaxial flow*. The exact diameter of that central sample core within the sheath stream is related to, among other things, the rate at which the sample is injected into the sheath stream; a 100 μm sheath stream may, depending on sample injection velocity, have a core width of perhaps 5–20 μm (Fig. 3.4). Because hydrodynamic focusing tends to confine the cell sample to this central core, there is little mixing of sample with sheath fluid (but diffusion of small molecules will occur). The reason that this type of coaxial sample flow suits flow cytometry

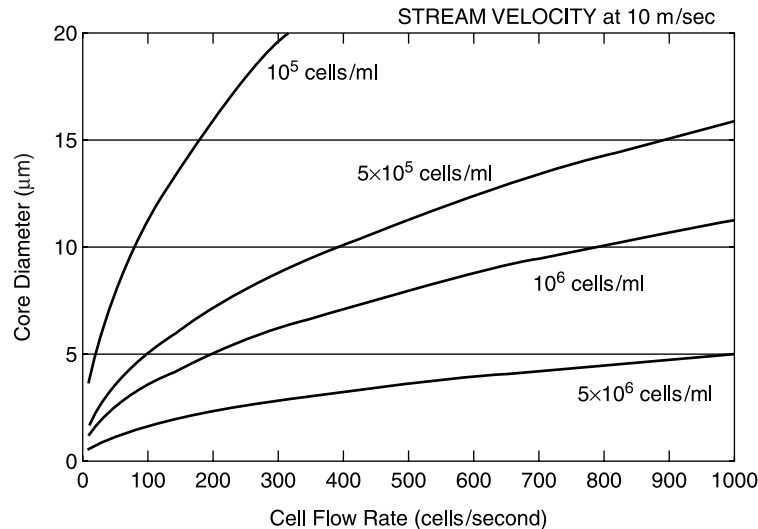


Fig. 3.4. When cells are pushed through the cytometer at faster rates, the sample core widens. Use of a more concentrated cell suspension allows a faster flow rate while maintaining a narrow core.

is that a nozzle or flow cell with a relatively wide orifice (50–250 μm) can be used to avoid blockage; the particles to be analyzed are nevertheless maintained in alignment in the narrow (say 5–20 μm) core so that they progress in single file down the center of the stream through the laser beam. This ensures uniform illumination as long as the central core is narrow and the illuminating beam is focused at the center of the sheath stream. It also ensures that, because the cells are stretched out at a distance from each other as rare beads along a gold chain, for the most part one particle is illuminated at a time.

We are now in a position to understand why radical changes in the rate at which the sample is pushed through the flow cell will cause changes in the resulting light signals. Changes in sample injection rate cause changes in the diameter of the core; when the size of the core increases, particles are no longer so tightly restricted in their position as they flow past the light beam, and illumination will be less uniform (Fig. 3.5). The size of the core often has critical impact in this way on DNA applications where precise analysis is important and nonuniform illumination causes nonuniform fluorescence. The stream diameter may be 100 μm, but if the illuminating beam has a width of 60 μm, then the core diameter needs to be considerably less than

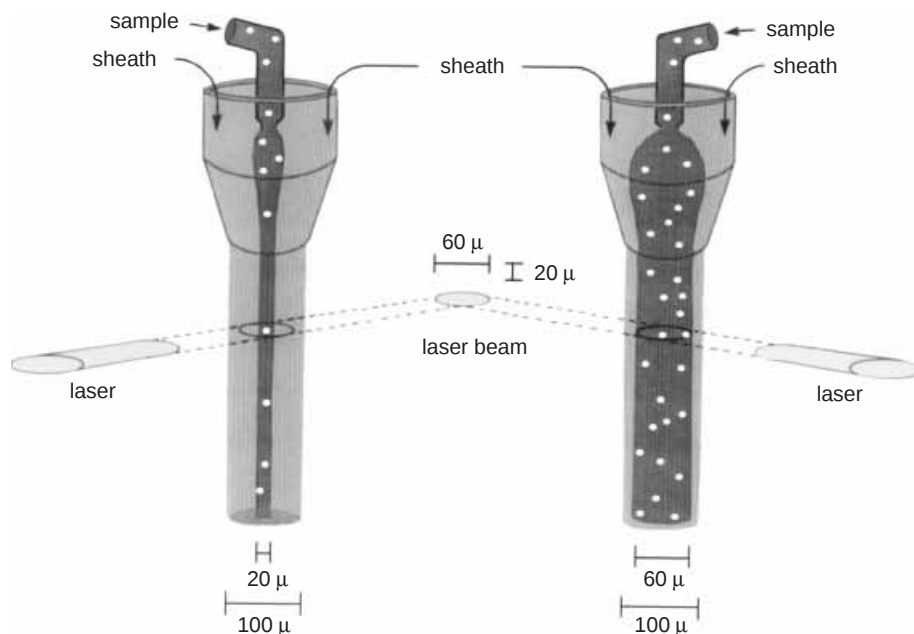


Fig. 3.5. The flow of cells within the core of sheath fluid through the analysis point in the illuminating beam. When the sample is injected slowly (left), the core is narrow and the cells flow one at a time through the center of the laser beam. When the sample is injected too rapidly (right), the core is wide (somewhat exaggerated in this drawing); the cells may be illuminated erratically because they can stray from the center of the beam. In addition, more than one cell may be illuminated at the same time.

60 μm to keep the cells at the center of the beam in order to ensure uniform illumination. In addition, with a wide sample core, two cells are more apt to be illuminated simultaneously, resulting in addition of their signals.

In some systems, the sheath stream with central sample core emerges from the orifice of a nozzle where it is then intersected by the light beam in the open air (a “jet-in-air” configuration). In other systems, the stream is directed (either upward or downward) through a narrow, optically clear, flow cell or chamber; the particles are illuminated by the light beam while they are still within this flow chamber. In still other systems, a nozzle forces the stream at an angle across a glass coverslip (Fig. 3.6). All systems have their advocates—the positive and negative considerations are based mostly on ideas about signal

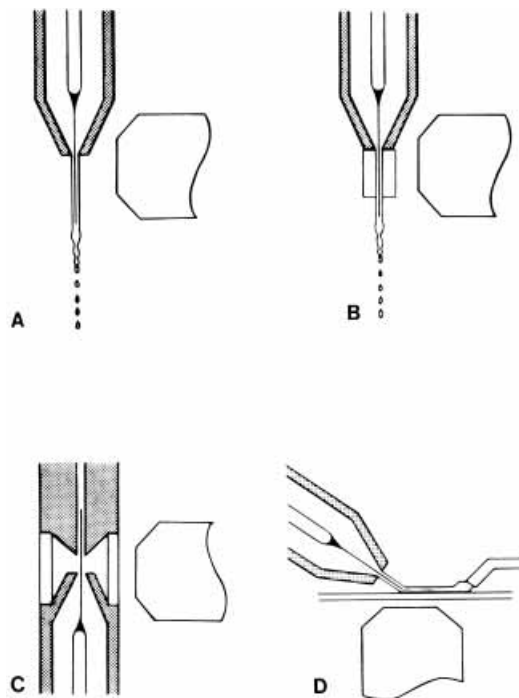


Fig. 3.6. Various types of flow chambers. **A** and **B** are designs used in sorting cytometers (in **A** the analysis point is in air after the stream has left the flow cell; in **B** analysis occurs within an optically clear region of the chamber itself). **C** and **D** are two designs for nonsorting cytometers (in **C** the stream flows upward through an optically clear region of the chamber; in **D** the stream is directed at an angle across a glass coverslip). Adapted from Pinkel and Stovel (1985).

noise, stream turbulence, and the control of drop formation for sorting. There is no clear favorite, and purchasers of commercial instruments usually base their choice of cytometer on factors other than flow cell design and then live with the design they get.

The main point of concern on a daily basis is the avoidance of blockage. Most flow cells in nonsorting cytometers have orifices with relatively wide dimensions (perhaps 150–250 μm in diameter) and are tolerant of large material; sorting instruments are more restricted in nozzle size. Although instruments can be modified to sort large cells, most commercial sorting cytometers operate with a stream diameter of between 50 and 100 μm . Cells larger than the nozzle orifice diameter will clog the nozzle. Furthermore, even if you think you have a

suspension of single cells, there will undoubtedly be some aggregates of much larger size.

DETECTION OF SIGNALS FROM CELLS

An optical bench is simply a table that does not wobble. A flow cytometer's optical bench may be visible at the back or may be incorporated behind a closed door; in either case, it provides a stable surface that fixes the light source and the light detectors in rigid alignment with the objects being illuminated. If the bench is moved, the light source, the light detectors, and the object of illumination will move in synchrony so that alignment between the three does not change. The reason that users of a flow cytometer should know about optical benches is, simply, to remind them that signals from a cell can vary beyond recognition if this alignment changes even slightly.

Figure 3.7 is a diagram of the components that sit on the optical bench

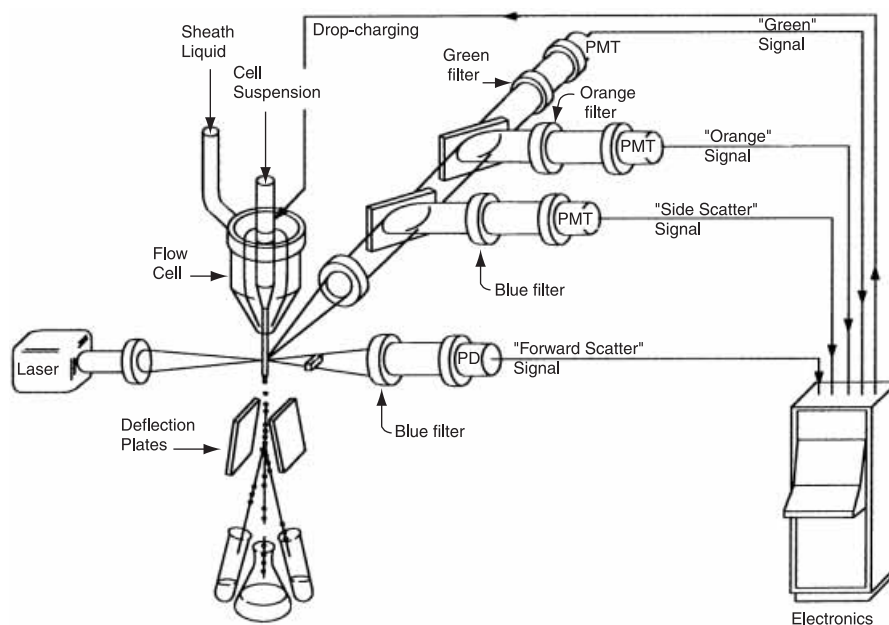
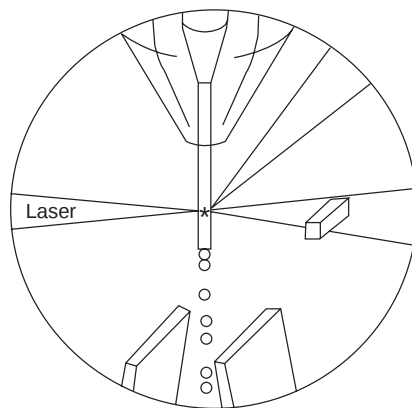


Fig 3.7. Components on the optical bench of a generalized "four-parameter" flow cytometer. (The drop charging, the deflection plates, and the drops moving into separate test tubes apply only to sorting cytometers [see Chapter 9] and not to benchtop instruments.) Adapted from Becton Dickinson Immunocytometry Systems.

bench of a flow cytometer. If we follow the light path from the beginning, we can see that the light, after it leaves the laser source, is focused through a lens into an elliptical beam of about 20 by 60 μm as it approaches the liquid stream. The stream of 50–150 μm diameter flows perpendicularly to the light beam. The alignment of the light beam and the stream must ensure that the stream is intersected by the light beam in such a way that the core of the stream (with its cells) is uniformly illuminated by the light. The point at which stream and light beam intersect (Fig. 3.8) is called the *analysis point*, *observation point*, or *interrogation point*. If the light beam and the stream are not perfectly and squarely aligned with each other, then cells within the stream will be erratically illuminated and will give off erratic light signals. Although the reasons for imperfect alignment may have to do with poor adjustment of the focusing lens or light source, the stability of the optical bench is, on the whole, reliable. On a day-to-day basis, poor alignment is much more likely to result from shifts in the fluid stream resulting from bubbles or partial obstruction.

Surrounding the analysis point are lenses that collect light as it emerges after its intersection with the cells in the stream. This emerging light constitutes the *signal*. It is focused onto photodiodes



★ = ANALYSIS POINT

Fig. 3.8. The analysis point. Alignment between the illuminating beam and fluid stream is critical in determining the characteristics of the resulting forward- and right-angle signals.

or photomultiplier tubes (the latter are more sensitive to dim light than the former; both can be called *photodetectors*) that convert the light signal into an electrical impulse. The intensity of the electrical impulse is proportional to the intensity of the light impinging on the detector.

The photodiode that sits in direct firing line of the illuminating beam receives signals that are responsible for much misunderstanding. Rather than simply detecting the amount of the illuminating beam (usually turquoise blue color) that gets through after bombarding a cell in the stream, this forward-angle photodetector is fitted with a so-called obscuration bar in front of it. The function of this narrow metal strip is to block the illuminating beam so that it cannot reach the detector. It may be wondered why we bother to place a photodetector in this position on the optical bench if we are then going to prevent any light from hitting it by blocking that light with a strip of metal. The critical point is that any light that has been bent as it passes through or around a cell will manage to avoid the bar, strike the photodiode, and generate a signal. Depending on the width of the bar, the angle of bending required to generate a signal is usually about 0.5° . This so-called forward scatter (FSC) or forward-angle light scatter (FALS) signal (defined as light of the same color as the illuminating beam that has been bent to a small angle from the direction of that original beam) is sometimes called a *size* or *volume* signal. It is undoubtedly related to the size and volume (mostly to the cross-sectional area) of the cell, but it is also related to other factors such as the refractive index of the cell. A cell with a refractive index very different from that of the surrounding medium will bend more light around the bar than will a cell with a refractive index closer to that of the stream. It is true that a large cell will bend more light than a small cell of the same refractive index; however, confusion inevitably results when the term *volume* is used carelessly to describe this forward-angle scatter.

For example, anyone used to looking at cells under a microscope will say that dead cells appear larger than living cells of the same type. It is therefore something of a surprise to find that dead cells appear “smaller” than living cells in a flow cytometer. Of course, they are not actually smaller; they simply give a dimmer forward scatter signal than living cells because they have leaky outer membranes, and the refractive index of their contents has for this reason

become more like the refractive index of the surrounding stream. They therefore bend less light into the FSC detector than a viable cell does. To give one more example (just to hammer the message home), erythrocytes, despite their uniform volume, give a broad range of forward scatter signals in a flow cytometer because their cross-sectional area varies depending on their orientation in the stream. However, anyone who remembers Fulwyler's experiments with red cells in a Coulter counter should not find this fact too surprising.

Since I mention Coulter volume measurements, I should add, for the sake of completeness, that some flow cytometers (most notably the now-obsolete Becton Dickinson FACS Analyzers) have a volume signal that is based on the Coulter-type measurement of electrical impedance. This is not in any way related to the FSC signal discussed in the paragraphs above. The Coulter-type volume signal is proportional to the volume of a particle (as well as to its electrical characteristics). The FSC signal is proportional to the cross-sectional area of a particle (as well as to its refractive index). Some state-of-the-art cytometers actually record both kinds of signals from particles; the ratio between the two varies for different types of particles, and this can be instructive. The take-home message is, therefore, that both of these kinds of signals give information about the physical characteristics of a particle, but neither tells much that we can count on about the true volume of that particle. The news, however, is not all bad. Although we may not know the exact biological meaning of a forward scatter signal, scatter characteristics can allow us to distinguish different classes of cells from each other, and this can, as we shall see, be useful.

In addition to this detector in the line of the laser beam (to detect FSC light), the standard configuration for photodetectors around the analysis point in a flow cytometer includes three, four, or more photomultiplier tubes at right angles to the illuminating beam. Because they are at the side and not in direct line of the illuminating beam, no light will hit these detectors unless something in the stream causes the illuminating beam to be deflected to the side or unless something in the stream is in itself a source of light in that direction. One of these detectors is usually fitted with a glass filter of the same color as the illuminating light (say, turquoise blue); it will register any illuminating (turquoise) light that is bounced to the side from the surface of a

cell in the stream. The rougher or more irregular or granular a particle is, the more it will scatter the illuminating beam to the side. The intensity of this so-called side scatter (SSC) light (defined as light of the same color as the illuminating beam that is scattered by a particle to an angle of 90° from the illuminating beam) is therefore related to a cell's surface texture and internal structure as well as to its size and shape. It is sometimes referred to as a *granularity signal* or an *orthogonal light scatter signal*. Granulocytic blood cells with their granules and irregular nuclei, for example, produce much more intense SSC than do the more regular lymphocytes or erythrocytes.

The several other photomultiplier tubes that sit at right angles to the illuminating beam are there to detect other colors of light (for example, green, orange, or red) that might be emitted by the cell. If the illuminating beam is blue, then there is no way that green, orange, or red light will emerge from the analysis point unless a cell in the stream is itself generating that green, orange, or red light. A cell can generate light either because it contains endogenous fluorescent compounds or because a scientist has stained it with a fluorescent stain. A cell stained with a fluorescent stain will, when illuminated from one direction by a beam of blue light, emit in all directions light of a different color (the color depending on the fluorescent stain used). It is this fluorescence that will be registered on one or another of the right-angle photomultiplier tubes.

The light coming out at right angles from the analysis point will be a mixture of light of the same color as the laser beam (SSC) and light of an assortment of other colors, depending on the fluorescent stains or endogenous molecules associated with the cell. Photodetectors are, unfortunately, relatively color blind; within their working range, they register all wavelengths of light more or less equally and give out electrical impulses that are proportional to intensity but provide no information about the color of the detected light. It is by restricting each photodetector, in some way, so that it receives only a particular color of light that we can associate the signal from that detector with a color and thereby obtain any specific knowledge about the color of our cell.

Therefore, in order for the signal from a particular photodetector to tell us what color a cell is, we need to use colored filters to make the photodetectors color specific. Photodetectors can be fitted with,

for example, green, orange, or red filters—depending on how many of these detectors are present in a particular instrument. The role of the filter is to ensure that each photodetector “sees” only light of the color transmitted through its own filter. If a green filter is in front of photodetector number one and an orange filter is in front of photodetector number two, then pd1 will respond with an electrical impulse if green light emerges from a cell at the analysis point, pd2 will respond with an electrical impulse if orange light emerges from that cell, both detectors will respond if white light emerges, and neither will respond if blue, or yellow, or no light appears. In this way, the signal from each photomultiplier tube can indicate the presence of a particular fluorochrome on a particle.

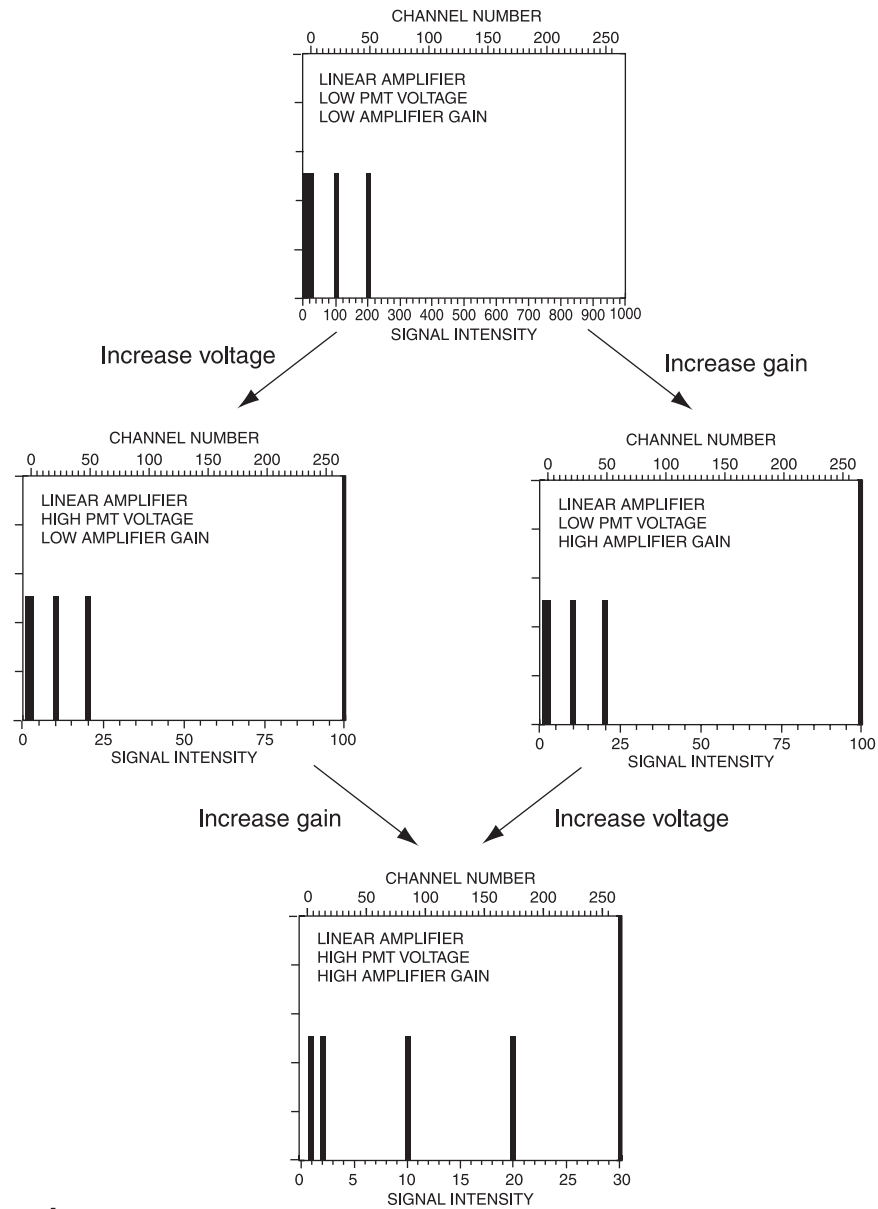
By way of recapitulation, before we move on to electronics, recall simply that, in the flow chamber, the sample with its suspended cells has joined the sheath fluid, and, within the core of that sheath stream, the cells have (with perfect orthogonal alignment) reached the intersection with the illuminating light beam. When the focused light beam hits the cell in the stream of fluid, the light is affected in several ways. It can be reflected, diffracted, and/or refracted; it can also be converted to a different color if it has been absorbed by a fluorescent chemical. The light that emerges after hitting a cell may register on one or more of the available photodetectors. The photodetectors each measure some specific aspect of the emerging light because of their positions, the color of their filters, or the presence of an obscuration bar. In a typical configuration, two of the photodetectors measure forward-angle scatter light and side scatter light in order to provide some information about the physical characteristics of the cell (sometimes called *size* and *granularity*); and three or more photodetectors are equipped with colored filters to provide information about the fluorescent light being emitted by the cells. These five or more characteristics registered on the photodetectors as each cell passes through the light beam are known as the *measured parameters* in the flow cytometry system. An instrument may, for example, be called a *three-parameter cytometer* or an *eleven-parameter cytometer* depending on how many photodetectors are arranged around the analysis point. Most commercial instruments now have a five- or six-parameter configuration. Once the light has hit the photodetectors, the light signals are converted into electrical signals.

ELECTRONICS

For many reasons, the circuit boards of a flow cytometer are things of beauty with which we are not going to concern ourselves. Integrated circuits (chips) do occasionally wear out; if they do, the symptoms can be most peculiar and difficult, even for a trained cytometer engineer, to diagnose (in our lab, we once had side scatter signals masquerading as green fluorescence). The message here is that you should get to know the engineer who services your instrument, and you should be nice to her at every possible opportunity. You will almost certainly need her expertise some day. In this section, however, I will describe just enough of the electronics so that you can understand the control that you can exert over the way the light signal given off by a particle reaches the computational circuitry for data analysis. Your ability to use these electronic controls correctly will have a significant influence on the interpretability of your data.

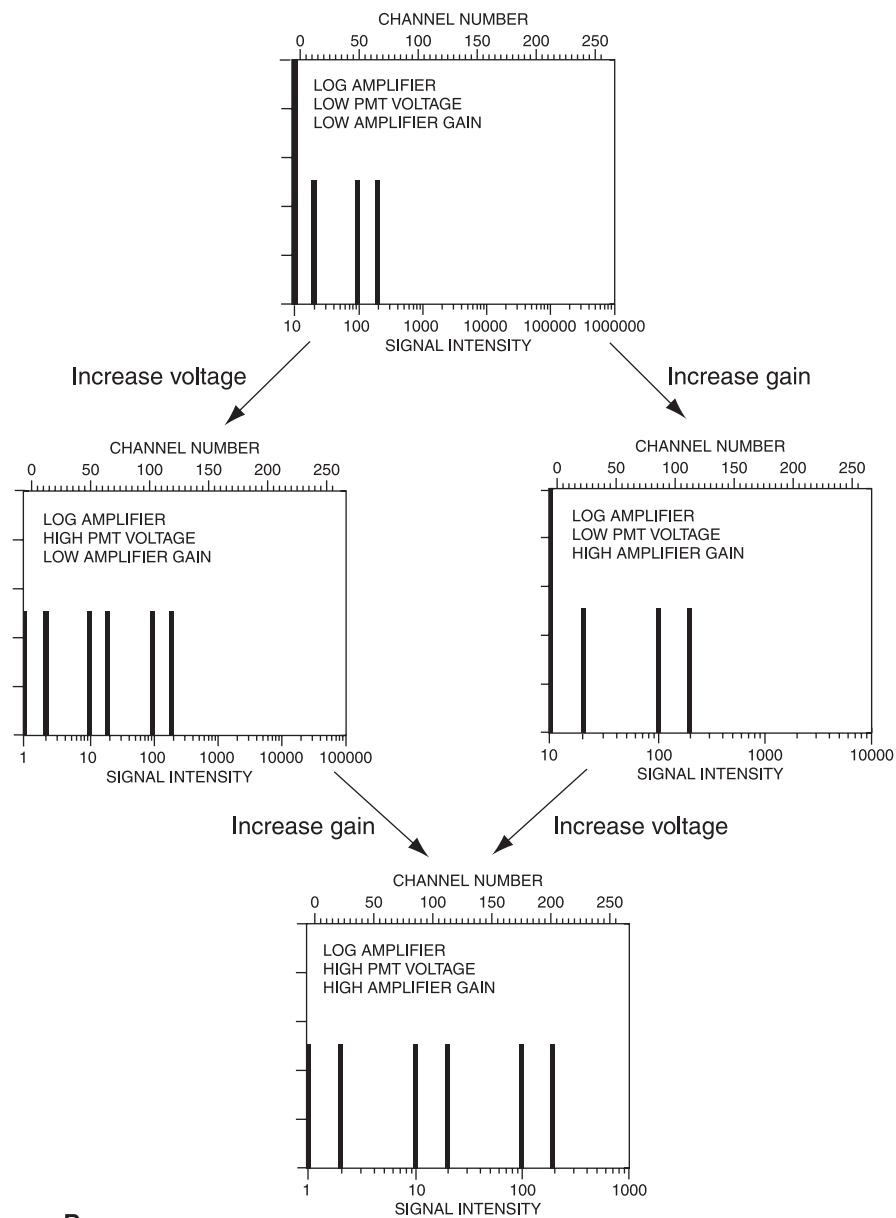
Photodetectors, as I have said, convert light signals into electrical impulses. The intensity of those electrical impulses are, within the limits of normal operation, related to the intensity of the light signals. However, the way in which the electrical impulses are then treated so that they are strong enough to be processed is open to user choice. Photomultiplier tubes (but not photodiodes) have voltages applied to them so that the cascade of electrons resulting from the original light impulse is converted into a sufficiently large current to be measured. Changing the voltage applied to a photomultiplier tube is one way we have of increasing or decreasing the current response of that tube (if we go too high or too low on the voltage, the response of the tube will no longer be proportional to the amount of light received). The second method we have at our disposal to increase or decrease the response to light is to change the amplification of that electrical current after it leaves the photodetector. Amplifiers can be either logarithmic or linear and can operate at varying gains. In general, we should be aware of the fact that a logarithmic amplifier allows us to look at light signals over a wide range of intensity; a linear amplifier may restrict our sensitive measurements to signals all in the same range.

Thus our two types of controls on the photodetectors are, one, the control of the voltage applied to photomultiplier tubes and, two, our



A.

Fig. 3.9. The effect of changes in the photomultiplier tube voltage and amplifier gain on the appearance of six signals with intensities in the relationship of 1:2:10:20:100:200 to each other. **A:** Linear amplification. **B:** Logarithmic amplification. (*continued on next page*)



B.

Fig. 3.9. (continued)

control over the amplifier. Our control of the amplifier can take the form of choice of log or linear amplification and also of choice of the precise gain applied. In addition, logarithmic amplifiers often allow an “offset” control, allowing the selection of the intensity range to be analyzed without changing the amplification. A comparison with log and linear graph paper is often helpful here. Figure 3.9 uses the graph paper analogy to indicate the effect these voltage and gain choices have on six theoretical signals, with intensities in the relationship of 1:2:10:20:100:200 to each other.

By looking at the “graph paper” scale on the lower horizontal axes, it can be seen that, with a linear amplifier, the origin is equal to zero; changing the voltage on the detector or changing the amplifier gain alters the range of signals that are displayed on scale. With a logarithmic amplifier, as with log graph paper, the origin is never equal to zero; changing the voltage changes the value assigned to the origin; changing the amplifier gain stretches or contracts the range of signals displayed. The gain setting used with a log amplifier is often referred to as, for example, *3 log decades full scale* or *4 log decades full scale*. What this means is that the top of the scale represents an intensity 10^3 or 10^4 times as bright as the bottom of the scale (think of log graph paper with 3 or 4 cycles). Many cytometers use log amplifiers that are fixed at a scale encompassing nominally 4 log decades. With other cytometers, the gain on the log amplifier can be varied by the operator to give a range of perhaps 2 to 5 log decades for the full scale. With a log amplifier, as with log graph paper, signals that are double in intensity relative to each other will be the same distance apart no matter where they are on the scale; in addition, distributions of signals with the same proportional variation around the mean (coefficient of variation [CV]) will look the same no matter where they appear on a log scale. Neither of these things is true with a linear amplifier or with linear graph paper.

Log amplifiers are usually employed to analyze fluorescence signals from cells with stained surface markers, because these cells often exhibit a great range of fluorescence intensities. Linear amplifiers are usually employed for analyzing the DNA content of cells, because the DNA content of cells does not normally vary by more than a factor of 2 (e.g., during cell division). Linear amplifiers may be used to analyze forward and side scatter signals, but practice here is apt to vary from lab to lab. With either linear or logarithmic amplification,

the choice of voltage or amplifier gain will depend on the range of intensities to be analyzed. The goal is to choose electronic settings so that the dimmest cells analyzed fall at the low end of the scale and the brightest cells fall at the top end of the scale.

Once the electrical signal from a photodetector has been amplified, its intensity is then analyzed and this value will be recorded by means of an analog-to-digital converter (an ADC). The role of an ADC is to look at a continuous distribution of signals and group (or bin) them into discrete ranges—much as you would need to do if you wanted to plot the heights of a group of people on a bar graph (histogram). For a height distribution histogram, the first bar might represent the number of people with heights (in old-fashioned feet and inches) between 4'9.5" and 4'10.5" ($4'10" \pm 0.5"$); the second bar, the number of people with heights between 4'10.5" and 4'11.5"; and so on (Fig. 3.10). Similarly, the ADC of a flow cytometer divides the electrical signals that it receives from each photodetector into discrete ranges.

Here we run into that term *channel* that flow cytometrists use so often. The ADCs on a flow cytometer are divided up into a discrete number of channels; the number of channels is usually 1024 (but may be 256 or even 65,536). Each channel represents a certain specific light intensity range (like the 17 channels each with a 1 inch range on our cadet height example). The signal from a cell is recorded in one or another channel depending on the intensity of that signal. It is the combination of photodetector voltage and amplifier gain that allows the user to set the intensity range represented by each of those channels. Using, for our example, a 256 channel ADC, we can now look back at the upper horizontal axis in each histogram in Figure 3.9. Having used the graph paper scale on the lower axis to plot intensity, we have now divided up the whole range of light intensity into 256 channels on the upper axis (numbered from 0 to 255). Varying the voltage and gain simply assigns a different intensity to each of the channels. The goal is to have the full range of channels encompass the full range of intensities relevant to a particular experiment. When the voltages and gains have been selected for a given experiment, the output data are then simply recorded by the cytometer electronics as light intensity on a scale of 0 to 255 (or 1023).

It is only by knowing something about the amplifier and voltage settings that you can know what relationship those values of 0 to 255 or 1023 bear to one another. For example, if we are using an ADC

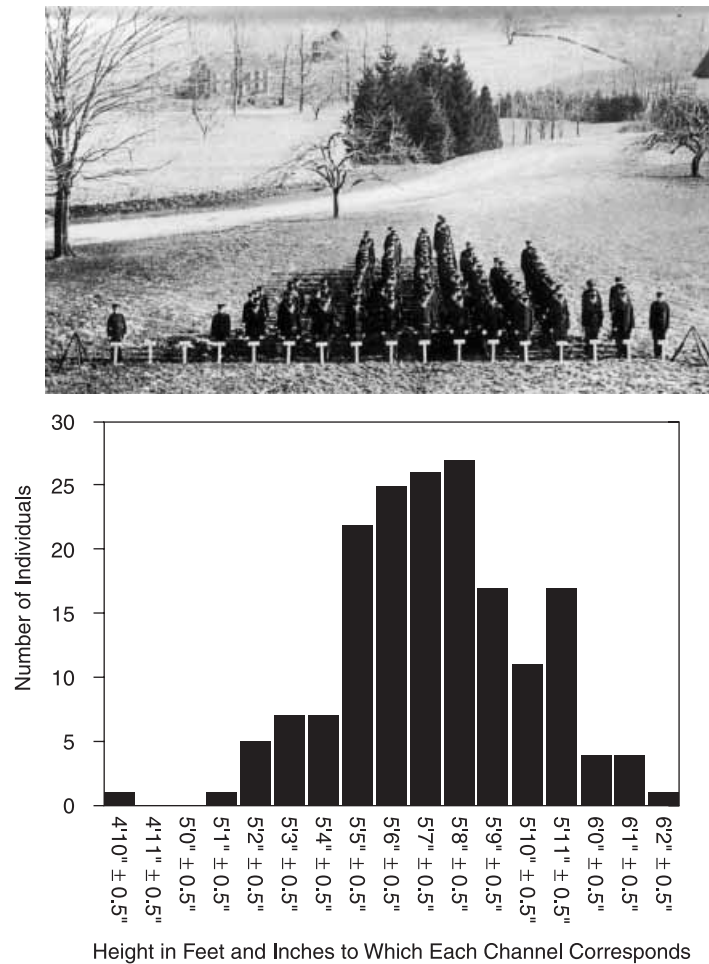


Fig. 3.10. World War One cadets from Connecticut Agricultural College arranged to form a height histogram. Photograph from A. Blakeslee (1914).

with 1024 channels and have selected a log amplifier with a gain that gives us 2 decades for the full scale, then we would know that a signal appearing in channel 700 has been given off by a cell that is 10 times as bright as a cell giving a signal in channel 188 (with a 2 decade full scale amplifier, every 512 channels represents a 10-fold increase in intensity; the entire 1024 channels represent two consecutive 10-fold increases in intensity = 10^2). If, on the other hand, the gain on the

log amplifier had been set to give us 4 log decades for the full scale, then a signal in channel 700 would come from a cell that is 100 times brighter than a cell giving a signal in channel 188 (on a 4 decade scale, every 256 channels represent a 10-fold increase in intensity; 512 channels represent a 10^2 -fold increase; the entire 1024 channels represent a 10^4 -fold increase). If we had been using a linear amplifier, then a signal in channel 700 would represent a cell 3.72 times brighter than one with a signal in channel 188 ($700/188 = 3.72$).

These examples should serve to emphasize that the numerical “read out” from a flow cytometer is relative and user-adjustable. A knowledge of instrument electronics and the settings used is required if we really want quantitative information about the relative brightness of signals from different cells; a simple channel number is not really enough. As an example, Figure 3.11 indicates real data (compare with Fig. 3.9 of model data) acquired with a mixed suspension of the same set of particles (beads of five different intensities) but with two different electronic settings (a linear amplifier in the histogram above and a log amplifier below). The channel numbers for the five peaks and the distribution of the peaks across the 1024 channels are very different in the two cases (next time you run beads or cells on a cytometer, try this yourself).

One other capability we have with cytometry electronics is the definition of a *threshold*. An electronic threshold is just like a threshold into a room: It defines an obstacle. Only cells giving signals greater than that obstacle will be registered on the cytometer ADC. The most common use of this threshold is in the definition of a forward scatter channel number. Only cells with a forward scatter signal brighter than the defined channel threshold will be registered by the cytometer. With the use of a forward scatter threshold, we can avoid problems that might come from dust, debris, and electronic noise in the system. The dim forward scatter signals from debris and noise are not bright enough to pass over the threshold, and “particles” of this type would be completely ignored. There are other ways of using a threshold (for instance, by using a red fluorescence threshold to exclude from an ocean water sample the signals from any particles that do not contain chlorophyll), but the use of a forward scatter threshold is by far the most common. Thresholds should be used with care; setting the threshold level too high can hide important data and can even make you think that you have no cells in your sample.

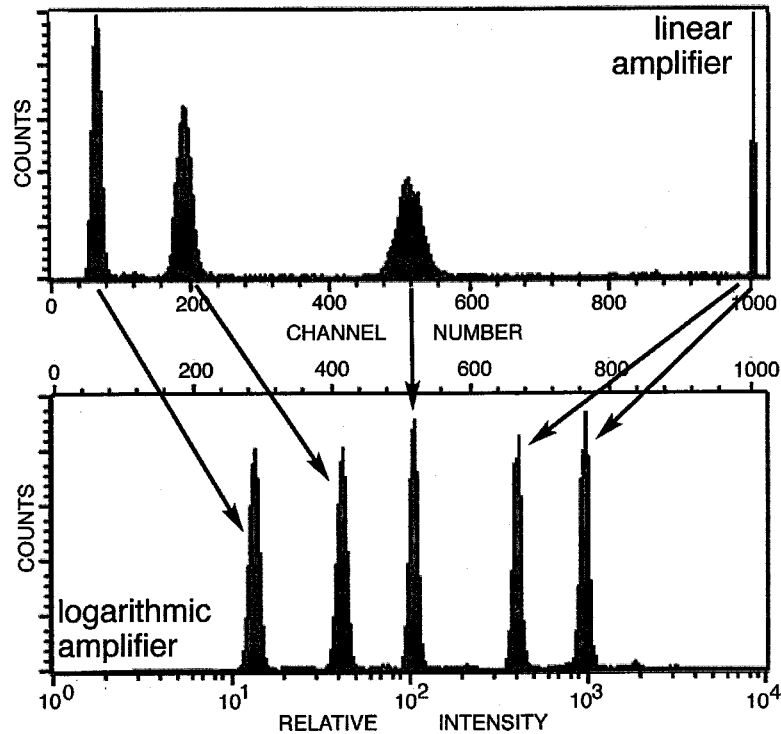


Fig. 3.11. Intensity signals from fluorescent beads (of five different intensities) acquired with linear amplification (top) and logarithmic amplification (bottom). Log amplification permits all five intensities to be “on scale” (that is, within the 1024 channel range). Additionally, the spread (the CV) and the peak height of the distributions for each bead are visually similar with a log but not with a linear amplifier. From Givan (2001).

By now, we should have a reasonably clear picture of the physical and electronic characteristics that form the basis for flow cytometric analysis. We have followed cells into the center of a stream flowing through a nozzle into a light path; we have seen how reflection, refraction, and fluorescence can generate light signals from those cells as they are hit by that light beam; we have accounted for registering of the light emerging from the cells onto one or another photodetector depending on the color or direction of that emerging light and the filters in front of the individual photodetectors; and we have described the way that the intensities of the light from cells registered

on each photodetector can be assigned to the discrete channels (1024) of an ADC. Therefore, for each cell that has flowed past the illuminating beam, we now have, simply, four or five or more numbers (depending on the number of photodetectors present) that describe that cell. Those four or five numbers (each on a scale of 0 to 1023) tell us the intensity of the FSC, SSC, and fluorescence (red, green, orange, and so forth) from that cell. Those numbers are, quite simply, the only information we now have about that cell. These are the facts that can be correlated with each other for data analysis. What we now do with the numerical data is up to the computer software and hardware available.

FURTHER READING

Chapter 2 in **Melamed et al.** and Chapters 1 and 4 in **Shapiro** are good general descriptions of cytometer characteristics.

Chapter 3 in **Melamed et al.** and Chapter 3 in **Van Dilla et al.** discuss hydrodynamics and flow chamber design in depth.

Chapter 2 in **Watson** has a good discussion of fluid flow dynamics and of the avoidance of coincidence events.

Chapter 5 in **Darzynkiewicz** covers flow cytometric optical measurements in general.